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14. ABSTRACT

This project focuses on the novel finding from our group that the formin protein, Drf3, is a signaling molecule positioned downstream from the EGF receptor that intersects with the tyrosine kinase Src in prostate cancer cells. Formins bind small GTPases and have been implicated in actin cytoskeletal remodeling. Evidence was presented in the original proposal that the EGFR Drf3 Src signaling circuit appears to traverse cholesterol-rich "lipid raft" membranes in prostate cancer cells. Lipid rafts are cholesterol-and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. We have hypothesized in this project, and in our published work in this area, that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of— and/or the types of—signals that flow through lipid raft microdomains. Several new lines of evidence consistent with our hypothesis have been produced in year 1 of the project and are described and summarized in this progress report.

15. SUBJECT TERMS
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INTRODUCTION

This project focuses on the novel finding from our group that the formin protein, Drf3, is a signaling molecule positioned downstream from the EGF receptor that intersects with the tyrosine kinase Src in prostate cancer cells. Formins bind small Rho-family GTPases and have been implicated in actin cytoskeletal remodeling. Evidence was presented in the original proposal that the EGFR→Drf3→Src signaling circuit appears to traverse cholesterol-rich "lipid raft" membranes in prostate cancer cells. Lipid rafts are cholesterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. We have hypothesized in this project, and in our published work in this area, that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of—and/or the types of—signals that flow through lipid raft microdomains.

BODY

In year 1 we have made significant progress on Task 2 and on preliminary studies for Tasks 1 and 4.

Task 2. **(i)** Identify the phosphorylation sites on Drf3 that are regulated by EGFR activation and **(ii)** determine the functional consequences of phosphorylation at these sites.

LNCaP cells were transiently transfected with FLAG-DRF3 constructs and the cells treated with 10 nM EGF or vehicle for 15 min. FLAG immunoprecipitates were generated from Triton-insoluble (lipid raft-enriched) fractions and proteins were subjected to reducing conditions by addition of DTT to a final concentration of 10mM and incubating at room temperature for 30 min, and secondly alkylating with 50mM iodoacetamide and incubating in the dark for 30 min. The immunoprecipitates were then resolved on a standard 8% SDS-PAGE gel and stained with SimplySafe Blue stain (Invitrogen) and then destained in water. Protein bands of interest were excised from gels and cut into approximately $1mm^3$ pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure. Samples were reconstituted in 5µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5µm C18 spherical silica beads into a fused silica capillary. The column was then attached to the HPLC system, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As each peptide was eluted they were subjected to electrospray ionization and then they entered into an LTQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern as calculated using the software program, Sequest (ThermoFinigan). The modification of 80 mass units to serine, threonine, and tyrosine was included in the

database searches to determine phosphopeptides. Each phosphopeptide that was determined by the Sequest program was also manually inspected to ensure confidence.

This mass spectrometry analysis identified a serine (624 position) on Drf3 that appeared to become phosphorylated in responsive to EGF (Figure 1).

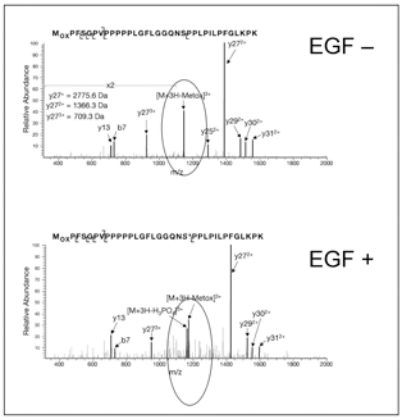

Figure 1. Peptide mass spectrum showing a phosphorylation event at serine 624 in Drf3 in the EGF treatment condition but not in the control condition (oval).

In order to confirm the effect of EGF on this residue, we attempted to make a phosphosite-specific antibody in rabbits using a synthetic phospho-peptide bordering the putative S624 phosphorylation site on both sides. We are currently in the process of testing this reagent now and we have accumulated significant amounts of encouraging data using it. We show some of these data here.

Using overexpressed mutant (S624A) and wild-type Drf3 proteins, we have been able to show conclusively that the P-Drf3 Ab recognizes the S624-P site with a high degree of specificity (Figure 2).

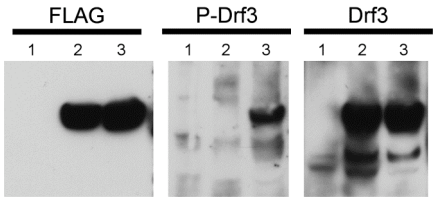

Figure 2. COS 7 cells were transfected with FLAG-Drf3^{WT} and the non-phosphorylatable FLAG-Drf3^{S624A} mutant. Lane 1 = whole cell lysate untransfected cells. Lane 2 = S24A mutant. Lane 3 = Drf3 WT. Labels show antibodies used for the blotting experiment.

We have also been able to confirm that the site becomes phosphorylated in response to EGF (Figure 3A), a result that is entirely consistent with our mass spectrometry analyses. In addition, we have been able to show that the P-Drf3 Ab recognizes ***endogenous*** Drf3 in prostate cancer cells (Figure 3B). Encouragingly, we have also been able to demonstrate that the native protein is phosphorylated in response to EGFR activation (Figure 3B). Interestingly, the phosphorylation site at S624 is cholesterol-sensitive, based on experiments in which membrane cholesterol is depleted using the cholesterol-binding compound, methyl-□-cyclodextrin (Figure 3C). This is an important finding because a major premise of our studies in this area is that ***cholesterol-sensitive***

post-translational modifications of proteins ***exist*** and that they direct signal transduction pathways into cholesterol-sensitive circuits.

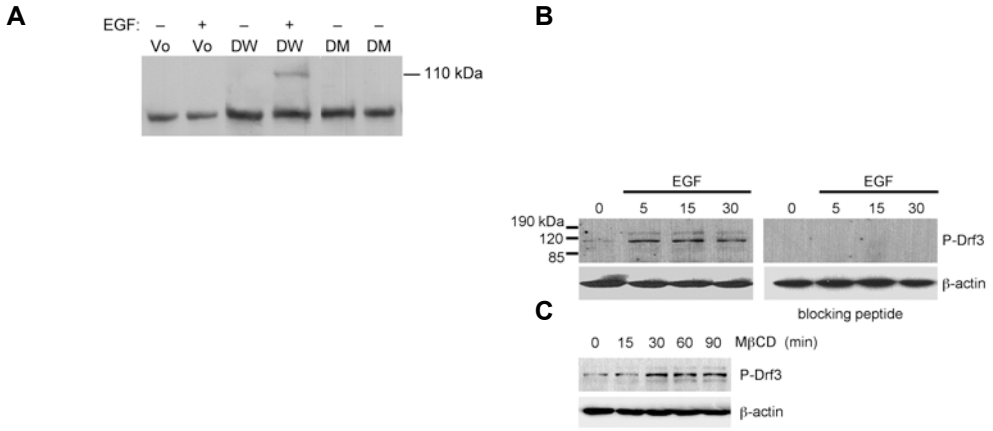

Figure 3. **A.** COS 7 cells were transfected with FLAG-Drf3^{WT} (DW) and the non-phosphorylatable FLAG-Drf3^{S624A} mutant (DM). Vo = vector-only control transfectants. Drf3 was immunoprecipitated with anti-Drf3 Ab and blotted with anti-P-Drf3 Ab generated by us. **B.** Identification of the EGF-dependent phosphorylation event at the S624 site on the endogenous Drf3 protein in PC3 prostate cancer cells in response to EGF. **C.** Increase in levels of phosphorylation at the S624 site in endogenous Drf3 following treatment of PC3 cells with the cholesterol-binding compound methyl-β-cyclodextrin (MβCD).

Another satisfying result has been that phosphorylation of endogenous Drf3 in the DU145 cell background can be manipulated by activation of Src, providing a direct link between Drf3 and Src that is consistent with our original hypothesis (Figure 4). In addition, consistent with our original identification of Drf3 as a protein that appears to undergo post-transcriptional modification in lipid raft membranes, blotting experiments indicate that P-Drf3 is highly enriched in the lipid raft membrane fraction of DU145 prostate cancer cells (Figure 4).

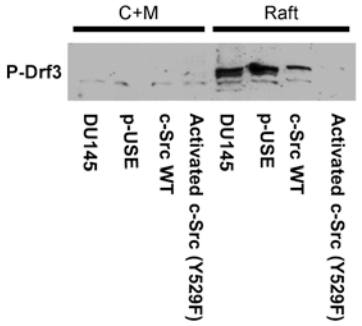

Figure 4. Suppression of phosphorylation of Drf3 at the S624 site in response to activation of Src. DU145 = control cells. P-USE = vector-only. C-Src WT = wild-type Src. C+M = cytoplasmic + nonraft membrane fraction. Raft = lipid raft-enriched membrane fraction.

Additional experiments indicate that DU145 cells are an excellent cell background to test the role of EGFR signaling through lipid raft microdomains. An ***unexpected discovery*** in this project has been the finding that fatty acid synthase (FAS), a metabolic "oncogene" involved in the synthesis of palmitic acid (a source of membrane lipids, lipid modification of proteins, and a precursor of cholesterol) can be induced to transit to lipid raft membranes in response to experimental manipulations in signal transduction pathways (treatment of cells with a PI3-kinase inhibitor, Figure 5). This effect was shown using two methods of lipid raft membrane enrichment (differential detergent extraction (Fig. 5A) and sucrose gradient centrifugation (Fig. 5B). Transit of FAS to raft membranes is not a result of apoptosis (Fig. 5C) and does not occur when cells are treated with apoptosis inducers (Fig. 5D). FAS is believed to be a cytosolic protein and is downstream from PI3K/Akt pathway signaling (Fig. 5E). Consequently, we believe these findings are highly novel and suggest a role for Src in alterations in lipid composition of tumor cell membranes.

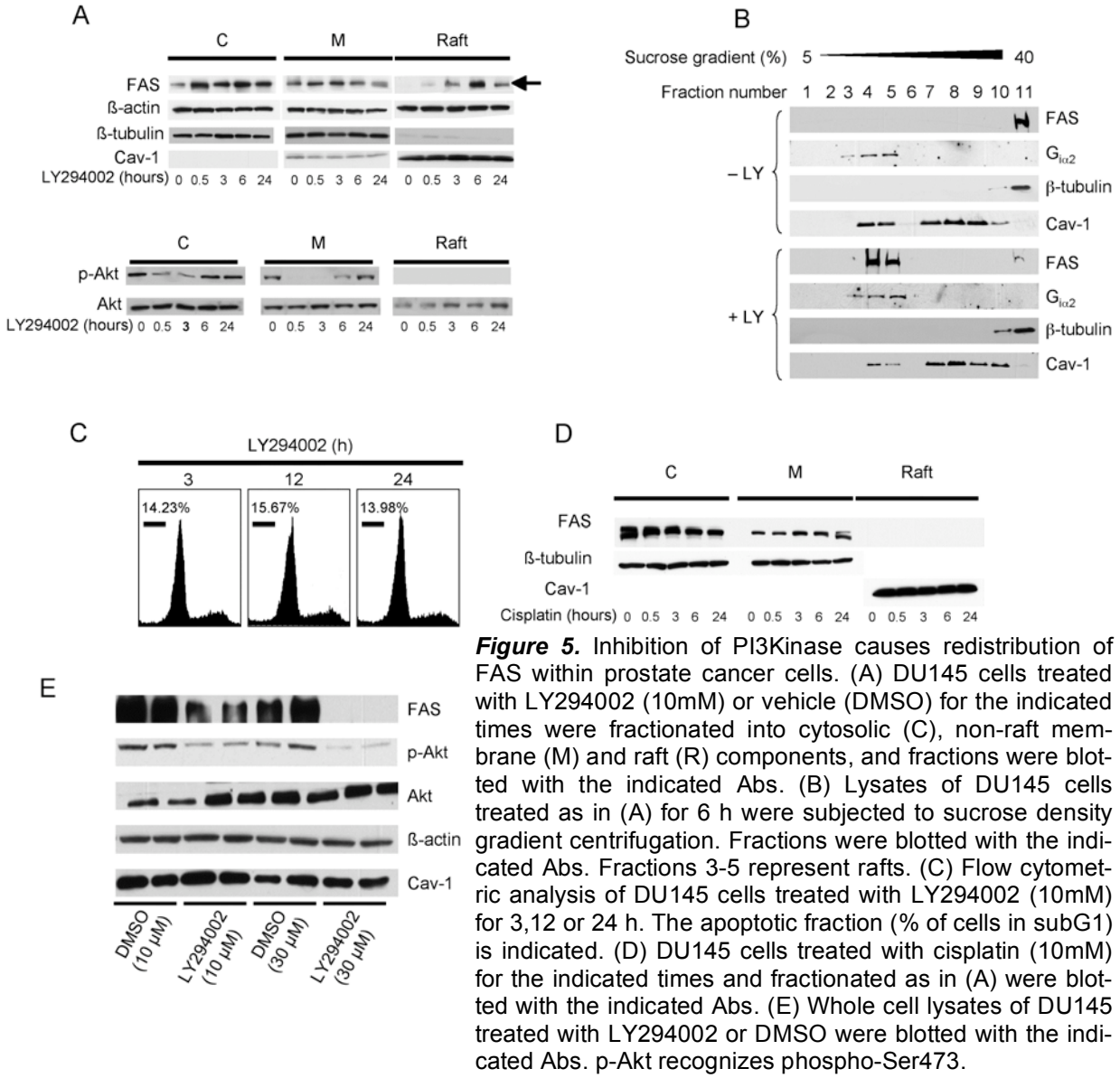

Figure 5. Inhibition of PI3Kinase causes redistribution of FAS within prostate cancer cells. (A) DU145 cells treated with LY294002 (10mM) or vehicle (DMSO) for the indicated times were fractionated into cytosolic (C), non-raft membrane (M) and raft (R) components, and fractions were blotted with the indicated Abs. (B) Lysates of DU145 cells treated as in (A) for 6 h were subjected to sucrose density gradient centrifugation. Fractions were blotted with the indicated Abs. Fractions 3-5 represent rafts. (C) Flow cytometric analysis of DU145 cells treated with LY294002 (10mM) for 3,12 or 24 h. The apoptotic fraction (% of cells in subG1) is indicated. (D) DU145 cells treated with cisplatin (10mM) for the indicated times and fractionated as in (A) were blotted with the indicated Abs. (E) Whole cell lysates of DU145 treated with LY294002 or DMSO were blotted with the indicated Abs. p-Akt recognizes phospho-Ser473.

These results led us to test the possibility that Src and FAS might physically interact. Co-immunoprecipitation experiments using DU145 cells have produced results consistent with this possibility (Figure 6A). Src in DU145 cells localizes predominantly to lipid rafts, indicating that this cell type is suitable for analyses of signaling from EGFR to Src through raft membranes (Fig. 6B). Importantly, inhibition of PI3-kinase in DU145 cells results in transient activation of Src in raft membranes (Fig. 6A). Finally, EGFR activation also results in enrichment of FAS in raft membranes, a result that allows us to connect the EGFR→Src pathway to the phenomenon of FAS localization to cholesterol-rich membrane sites.

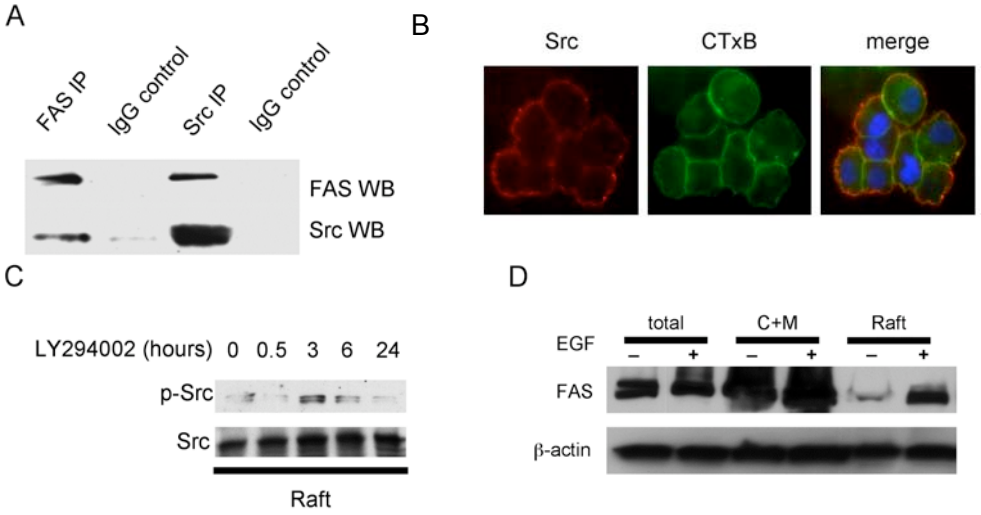

Figure 6. Src and FAS physically interact, and activation of Src is coincident with FAS relocalization to rafts. **(A)** c-Src & FAS were immunoprecipitated from DU145 whole cell lysates using anti-c-Src mAb and anti-FAS rAb. IP eluates were blotted using the indicated Abs. An irrelevant antibody was used as an IP control. **(B)** IF using FITC-CTxB, and c-Src antibody (Cy3) in DU145. **(C)** Raft fractions from DU145 treated with LY294002 (10μM) for the indicated times were blotted with pY416-Src antibody (activated Src) (upper panel) and total Src (lower panel). The data show enrichment of p-Src in rafts after 3 h of LY294002 treatment. **(D)** Equal amounts of whole lysates or C+M and raft fractions of DU145 cells treated with EGF (50 ng/ml, 30 min) were blotted with Abs to FAS and β-actin.

Collectively, these results have given us the tools to determine the biological consequences of (1) Drf3 ablation using RNA silencing; and (2) mutating the S624 phosphorylation site to a residue that cannot be phosphorylated (alanine). These are experiments proposed in ***Task 1*** and ***Task 4*** in the original application.

KEY RESEARCH ACCOMPLISHMENTS

- In year 1 we have experimentally verified that Drf3 is a lipid raft protein that lies downstream of the EGFR in prostate cancer cells.
- We have identified a novel EGFR-responsive phosphorylation site on this protein and made and successfully tested an Ab that recognizes the phosphorylated form of the protein.
- We have also laid the groundwork for extensive study in year 2 of EGFR→Drf3→Src signaling in the DU145 cell background.

These experiments have also led to the novel and unexpected discovery that EGFR activation and activation of Src lead to transient association of the metabolic oncogene, FAS, to lipid raft membranes. FAS is of direct relevance to prostate carcinogenesis and progression because FAS is overexpressed in the human disease and in animal models of prostate cancer.

REPORTABLE OUTCOMES

To date, three publications attributable in whole or part to funding from this grant have been published.

Lutchman, M., Solomon, K.R., and **Freeman, M.R**. Cholesterol, cell signaling and prostate cancer. In: Prostate Cancer: Novel Biology, Genetics and Therapy. Chung, L.W.K., Isaacs, W.B., and Simons, J.W. Second edition. Humana Press (In press).

Freeman, M.R., Cinar, B., Kim, J., Mukhopadhyay, N.K., Di Vizio, D., Adam, R.M., and Solomon, K.R. (2006) Transit of hormonal and EGF receptor-dependent signals through cholesterol-rich membranes. Steroids (Epub ahead of print—Dec 13).

Hager, M., Solomon, K.R., and **Freeman, M.R.** (2006) The role of cholesterol in prostate cancer. Current Opinion in Clinical Nutrition and Metabolic Care 9:379-385.